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Glucocorticoids protect renal mesangial cells from apoptosis by increasing cellular sphingosine-1-phosphate

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Neutral ceramidase (NCDase) and sphingosine kinases (SphKs) are key enzymes regulating cellular sphingosine-1-phosphate (S1P) levels. In this study we found that stress factor-induced apoptosis of rat renal mesangial cells was significantly reduced by dexamethasone treatment. Concomitantly, dexamethasone increased cellular S1P levels, suggesting an activation of sphingolipid-metabolizing enzymes. The cell-protective effect of glucocorticoids was reversed by a SphK inhibitor, was completely absent in SphK1-deficient cells, and was associated with upregulated mRNA and protein expression of NCDase and SphK1. Additionally, *in vivo* experiments in mice showed that dexamethasone also upregulated SphK1 mRNA and activity, and NCDase protein expression in the kidney. Fragments (2285, 1724, and 1126 bp) of the rat NCDase promoter linked to a luciferase reporter were transfected into rat kidney fibroblasts and mesangial cells. There was enhanced NCDase promoter activity upon glucocorticoids treatment that was abolished by the glucocorticoid receptor antagonist RU-486. Single and double mutations of the two putative glucocorticoid response element sites within the promoter reduced the dexamethasone effect, suggesting that both glucocorticoid response elements are functionally active and required for induction. Our study shows that glucocorticoids exert a protective effect on stress-induced mesangial cell apoptosis *in vitro* and *in vivo* by upregulating NCDase and SphK1 expression and activity, resulting in enhanced levels of the protective lipid second messenger S1P.

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Sphingomyelin is the most abundant sphingolipid in cellular membranes, in which it serves not only as a structural component, but also as a precursor for various sphingolipid signaling molecules, including ceramide and sphingosine-1-phosphate (S1P). These two bioactive lipids regulate a variety of physiological and pathophysiological processes such as growth and differentiation, cell migration and adhesion, apoptosis, and inflammatory reactions.[1–5]

Upon exposure of cells to various stress factors, including proinflammatory cytokines, sphingomyelinases are activated and hydrolyze sphingomyelin to generate ceramide. Ceramide is further degraded by ceramidases to sphingosine that, in turn, serves as a substrate for sphingosine kinases to generate S1P. In contrast to ceramide and sphingosine, both of which can trigger an apoptotic cell response, S1P exerts an effect to counter-balance this response and stimulates cell proliferation and protects cells from apoptosis. This has led to the hypothesis of a cellular sphingolipid rheostat in which ceramide and S1P counter-regulate each other's functions.[1,3,6]

As sphingosine is not produced by *de novo* synthesis,[7] the activity of ceramidases (*N*-acylsphingosine amidohydrolase, ASAH) is crucial for limiting the ceramide-induced signaling as well as for the generation of sphingosine and S1P.

Ceramidases are classified into three different groups according to their catalytic pH optimum, namely acid, alkaline, and neutral ceramidases.[8] The acid ceramidase is localized in the lysosomes[9] and its genetic mutation causes Farber's disease, a disorder in which ceramide excessively accumulates in lysosomes.[10] The alkaline ceramidase is localized in the endoplasmic reticulum and Golgi, but its physiological role is hardly understood.[11] The neutral ceramidase (NCDase) has been cloned from various species[8] and the primary sequence among the species is highly conserved. A predominant localization of NCDase at the plasma membrane has been reported.[12,13] To date, only little is known about the transcriptional and posttranscriptional regulation of NCDase. It has been shown that proinflammatory cytokines, such as interleukin-1β and tumor necrosis

factor-α (TNF-α), increased the NCDase expression in hepatocytes[14] and mesangial cells,[15,16] whereas nitric oxide decreased the NCDase expression levels by a proteasomal degradation route.[17,18] In addition, under diabetic conditions the expressions and activities of NCDase and sphingosine kinase were increased in kidney, and increased S1P concentrations were proposed to trigger for hyperproliferation of mesangial cells in diabetic glomeruli.[19] Recently, the promoter of the mouse *asah 2* gene was cloned[20] and revealed potential binding sites for GATA-2, C/EBP, and HNF3, although the functionality of these sites remained undetermined.

Sphingosine kinases (SphKs) are the critical enzymes directly generating S1P. SphKs exist as two subtypes,[21] SphK1 and SphK2, although the differential physiological functions of these two subtypes are presently unclear. At least, SphK1 has been attributed a key function in cell proliferation and migration, and an upregulation of SphK1 mRNA is reported for several forms of tumor tissues and cell lines.[21–24] In contrast, SphK2 rather exerts a pro-apoptotic function based on cellular transfection experiments.[25]

In this study, we used rat renal mesangial cells and showed that glucocorticoids protect mesangial cells from stress-induced apoptosis. This protective effect is because of a coordinated upregulation and activation of NCDase and SphK1 that resulted in an increased cellular S1P level. In addition, in kidneys of dexamethasone-treated mice, SphK1 expression and activity, and NCDase expression were enhanced in glomeruli or whole kidney extracts. Furthermore, we showed that the rat NCDase promoter contains two functional glucocorticoid-response element (GRE) sites that contribute to the enhancing effect of glucocorticoids on NCDase gene transcription.

RESULTS

Dexamethasone protects renal mesangial cells from apoptosis

Stimulation of rat renal mesangial cells with staurosporine or TNF-α plus cycloheximide is well known to induce apoptosis.[15,26–28] Accordingly, increased DNA fragmentation was observed after 16 h of stimulation with these factors (Figure 1a) when using a DNA fragmentation enzyme-linked immunosorbent assay. In the presence of dexamethasone, which exerts its effect by binding to the glucocorticoid receptor (GR) that is expressed in mesangial cells (Figure 1a, inset), a significantly reduced DNA fragmentation occurred (Figure 1a). To quantify the amount of apoptotic cells, we additionally performed flow cytometry to determine the amount of cells in the subG1 phase, which is considered to represent apoptotic cells. In untreated cells, we found $5.9 \pm 1.5\%$ apoptotic cells that increased to $50 \pm 2\%$ after 16 h of staurosporine treatment. The inhibiting effect of dexamethasone was reversed in the presence of the GR antagonist RU-486[29] (Figure 1b), indicating that the protective effect is mediated by the GR receptor. Furthermore, a panel of other glucocorticoids, including betamethasone, hydrocortisone, fludrocortisone, and the mineralocorticoide aldosterone, which also bind to the GR, exerted a similar inhibitory effect on staurosporine-induced apoptosis (Figure 1c). Interestingly, the protective effect of glucocorticoids on staurosporine-triggered apoptosis was also reversed by a SphK1 inhibitor, SKI II[22] (Figure 1d), suggesting that SphK1 is involved in the protective action of glucocorticoids. In an additional approach, we used mouse mesangial cells isolated from either wild-type or SphK1 knockout mice. In wild-type mesangial cells, staurosporine triggered a marked increase of apoptosis, which was again reduced in the presence of dexamethasone (Figure 2). In SphK1 knockout cells, the staurosporine-induced apoptosis was even higher when compared with the wild-type cells, which is consistent with the hypothesis that SphK1 is a protective enzyme. Remarkably, dexamethasone had no protective effect in these SphK1 knockout cells (Figure 2).

Dexamethasone stimulates S1P generation in rat renal mesangial cells

As S1P has recently been attributed an anti-apoptotic capacity,[3,5,30] we speculated that glucocorticoids may alter the cellular levels of this sphingolipid second messenger. To this end, cellular S1P levels were quantified by mass spectrometry. As seen in Figure 3, the cellular S1P concentration was very low, but significantly increased upon dexamethasone treatment and was abolished in the presence of RU-486, thus suggesting that either the S1P-generating pathway is activated or the S1P-metabolizing pathways are inhibited.

Therefore, we next analyzed the activity of SphK1 upon dexamethasone treatment. Stimulated cell lysates were taken for an *in vitro* kinase activity assay using sphingosine as a substrate. Both concentrations of dexamethasone tested (that is, 100 and 300 nM) led to a time-dependent increase in SphK1 activity. In parallel to the increased SphK1 activity (Figure 4a), SphK-1 protein expression was also increased (Figure 4b) and this was preceded by an upregulated mRNA expression of SphK1 (Figure 4c).

As sphingosine is normally not stored in the cell, we analyzed whether the sphingosine-generating enzyme, that is, the NCDase, is also regulated by glucocorticoids. As seen in Figure 5, NCDase mRNA (Figure 5a) and protein expression (Figure 5b) is upregulated by dexamethasone treatment. Two forms of NCDase protein have been described in rat kidney: a 133 kDa Golgi form and a 113 kDa endoplasmic reticulum form.[31] Notably, dexamethasone only upregulated the 113 kDa endoplasmic reticulum form. In contrast, the acid ceramidase was not upregulated by dexamethasone (data not shown).

In a next step we analyzed whether the glucocorticoid-mediated upregulation of the ceramidase/SphK1 pathway is also occurring *in vivo*. To this end, we treated mice for 24 h with dexamethasone (10 mg/kg) and harvested kidneys and prepared protein extracts from whole kidney and also isolated glomeruli from these kidneys for RNA extraction.

As seen in Figure 6a, SphK1 mRNA expression is significantly enhanced in the glomeruli of the dexamethasone-treated mice. In addition, when measuring SphK1 activity in whole kidney extracts, an increase in the same range was found (Figure 6b). Finally, NCDase protein expression in whole kidney lysates was also upregulated (Figure 6c).

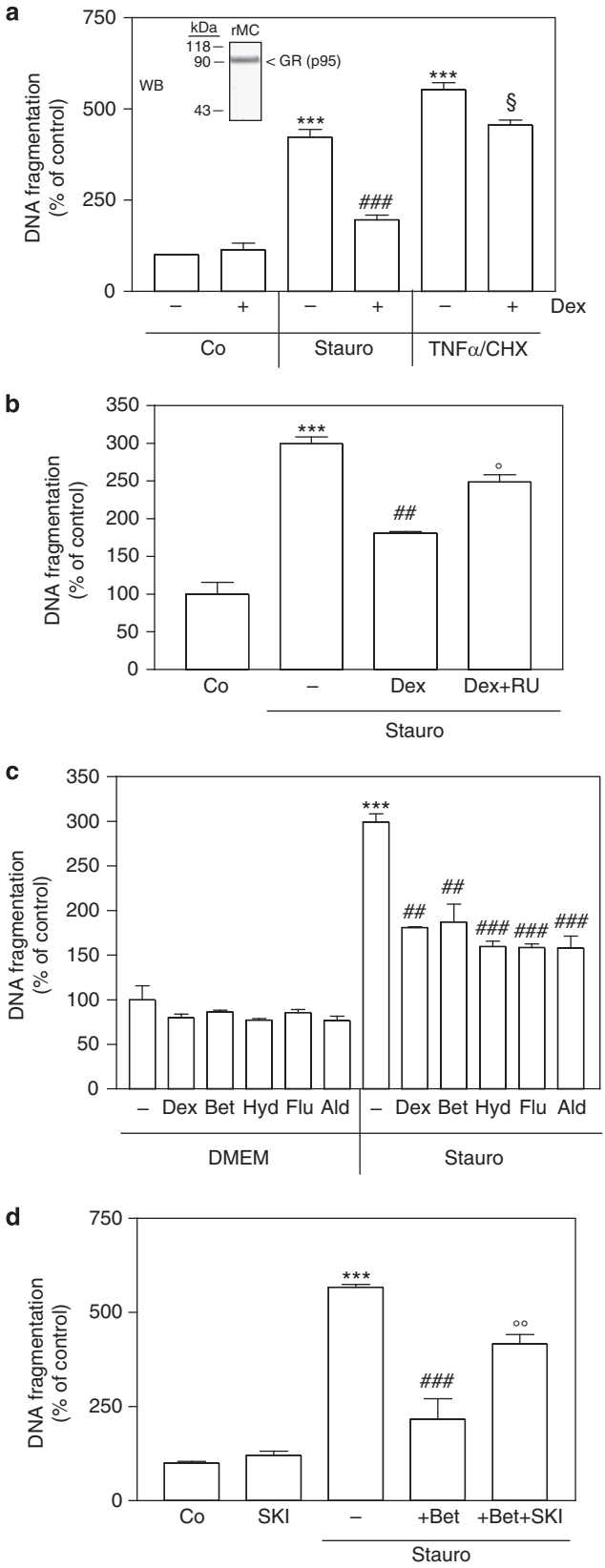

Figure 1 | Effect of dexamethasone on stress-induced DNA fragmentation in renal mesangial cells. (**a**) Quiescent mesangial cells were preincubated for 1 h with either vehicle (−) or dexamethasone (dex, 300 nM, +) before stimulation for 16 h with either vehicle (Co), staurosporine (stauro, 30 nM), or tumor necrosis factor-α (TNF-α; 1 nM) plus cycloheximide (10 μg/ml) (TNF-α/CHX). The inset shows the protein expression of the 95 kDa glucocorticoid receptor (GR) in lysates of rat mesangial cells (MCs) by western blot analysis. (**b**) Cells were treated for 16 h with stauro (30 nM), stauro + dex (300 nM), or stauro + dex + RU-486 (RU, 1 μM). (**c**) Cells were treated with vehicle (Co) or stauro (30 nM) in the absence (−) or presence of dex, betamethasone (bet), hydrocortisone (hyd), fludrocortisone (flu), or aldosterone (ald) (all at 300 nM). (**d**) Cells were treated for 16 h with vehicle (Co), SKI II (SKI, 10 μM), stauro (30 nM), stauro + bet (300 nM), or stauro + bet + SKI (10 μM). DNA fragmentation was analyzed as described in the Materials and Methods section. Results are expressed as percentage of control values and are means ± s.e.m. ($n = 6$ in **a**, $n = 3$ in **b**–**d**). $^{***}P < 0.001$ considered statistically significant when compared with the unstimulated control values; $^{\#\#\#}P < 0.001$ when compared with the staurosporine-stimulated values; $^{\S}P < 0.05$ when compared with the TNF-α plus cycloheximide-stimulated values; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ when compared with the stauro plus dex/bet-stimulated values.

Dexamethasone exerts an effect through GREs in the rat NCDase promoter sequence

We cloned a 2258-bp fragment of the rat NCDase promoter and two shorter fragments of 1724 and 1126 bp size, respectively. These fragments were fused to a luciferase

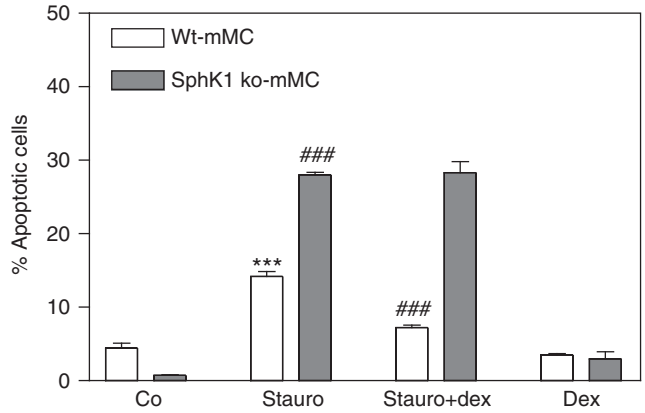

Figure 2 | Effect of dexamethasone on stress-induced apoptosis in sphingosine kinase 1 (SphK1) knockout mouse mesangial cells. Mesangial cells isolated from either wild-type C59BL6 mice (wt-mMC, open bars) or SphK1-deficient mice (SphK1ko mMC, closed bars) were treated for 16 h with either vehicle (Co), staurosporine (stauro, 30 nM), staurosporine (30 nM) plus dexamethasone (300 nM) (stauro + dex), or dexamethasone alone (300 nM). Cells were taken for flow cytometry to determine the number of cells in the sub G1 phase. Results are expressed as percentage of apoptotic cells and are means ± s.d. ($n = 3$). $^{***}P < 0.001$ considered statistically significant when compared with the vehicle-treated wt values; $^{\#\#\#}P < 0.001$ when compared with the staurosporine-stimulated values.

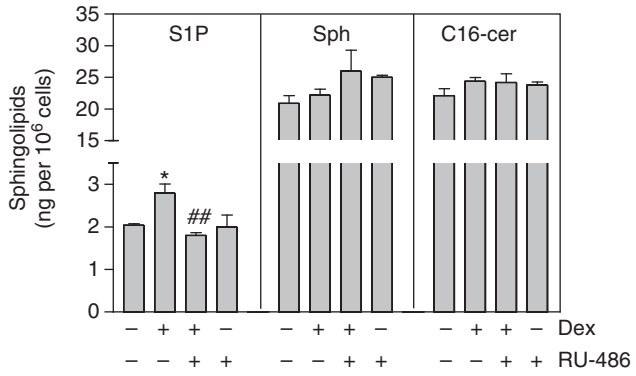

Figure 3 | Effect of dexamethasone on cellular sphingosine 1-phosphate (S1P), sphingosine (Sph), and C16-ceramide (C16-cer) concentrations in renal mesangial cells. Quiescent mesangial cells were stimulated for 16 h with either vehicle (–), dexamethasone (Dex, 300 nM), or dexamethasone plus RU-486, 1 μM). The cellular sphingolipid concentrations were quantified by liquid chromatography-coupled mass spectrometry (LC/MS/MS) as described in the Materials and Methods section. Results are means ± s.d. ($n = 3$). $^{*}P < 0.05$ considered statistically significant when compared with the control samples; $^{\#\#}P < 0.01$ significant when compared with the dex-treated samples.

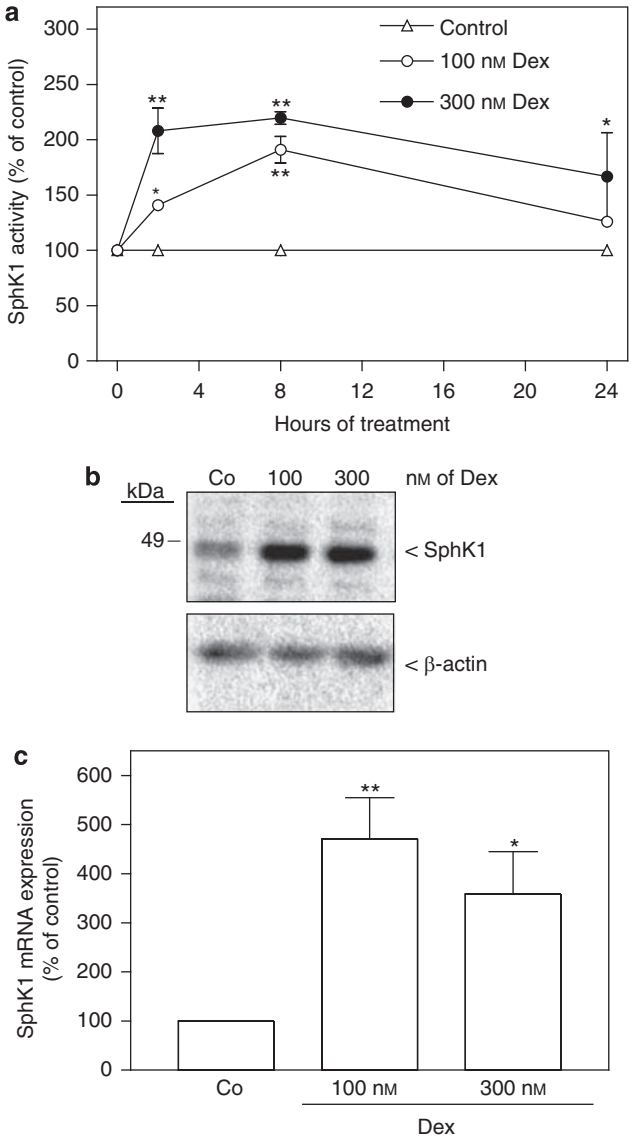

Figure 4 | Effect of dexamethasone on sphingosine kinase 1 (SphK1) activity, mRNA, and protein expression in renal mesangial cells. (**a**) Quiescent mesangial cells were stimulated for the indicated time periods with either vehicle (triangles), or 100 nM (Dex; open circles), or 300 nM (Dex; closed circles) of dexamethasone (Dex). Protein extracts containing equal amounts of proteins were taken for an *in vitro* SphK1 activity assay as described in the Materials and Methods section. Data are expressed as percentage of control and are means ± s.e.m. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ considered statistically significant when compared with the vehicle-stimulated values. (**b, c**) Cells were stimulated for 24 h with either vehicle (Co) or 100 nM or 300 nM of dexamethasone (dex). Thereafter, protein (**b**) or RNA (**c**) was extracted. Protein extracts were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and subjected to western blot analysis using a specific antibody against SphK1 at a dilution of 1:2000 or against β-actin at a dilution of 1:1000. Data in **b** are representative of three independent experiments. RNA was subjected to quantitative PCR analysis using specific primers of rat SphK1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data in **c** are expressed as percentage of control values and are means ± s.e.m. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ considered statistically significant when compared with the vehicle-stimulated values.

reporter construct and transfected into normal rat kidney fibroblast (NRK) cells and rat renal mesangial cells. As seen in Figure 7a, in NRK cells, dexamethasone was able to stimulate the activity of all promoter fragments. In the presence of RU-486, the dexamethasone-triggered NCDase promoter activation was completely abolished in all fragments (Figure 7a). A similar stimulating effect of dexamethasone and reversing effect of RU-486 was also observed in rat mesangial cells (Figure 7b). As rat mesangial cells show a very low transfection efficiency, we used NRK cells for further studies. Other glucocorticoids were tested such as beta-methasone, hydrocortisone, and fludrocortisone. All these drugs exerted a similar stimulating effect on NCDase promoter activity (Figure 8). As the promoter sequence contained two putative GREs at position –1337 bp (GRE1) and at –890 bp (GRE2), we generated deletion mutants of the 1724 bp fragment. When the GRE1 site was deleted (ΔGRE1), dex could still activate the promoter although to a significantly reduced degree (Figure 9). When both sites were deleted (ΔGRE1 + 2), the dex effect was completely abolished (Figure 9), suggesting that both sites are essential for the glucocorticoid effect.

DISCUSSION

In the past decades, glucocorticoids have been extensively studied because of their potent and versatile pharmacological profile. Many studies have meanwhile proven that glucocorticoids have either pro- or anti-apoptotic capacities depending on the tissue and cell type that they exert an effect upon. An induction of apoptosis is mainly observed in hematopoietic and immune cells.[32] In contrast, a death protective effect has been characterized for epithelial cells, fibroblasts, endothelial cells, and hepatocytes.[33–35] Although dexamethasone was shown to suppress (1) the upregulation of proapoptotic

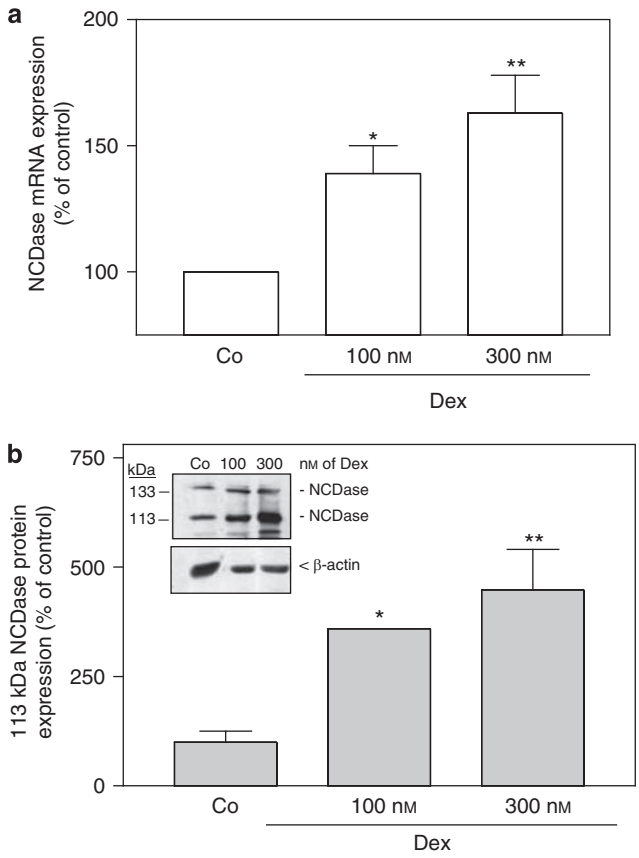

Figure 5 | Effect of dexamethasone on neutral ceramidase (NCDase) mRNA and protein expression in renal mesangial cells. Quiescent rat renal mesangial cells were stimulated for 20 h with either vehicle (Co) or the indicated concentrations of dexamethasone (dex). Thereafter, RNA or protein was extracted. (**a**) The RNA was subjected to quantitative PCR analysis of NCDase. (**b**) Protein extracts were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and subjected to western blot analysis using a polyclonal antiserum against NCDase at a dilution of 1:1000. Bands corresponding to the 113 kDa NCDase and β-actin were densitometrically evaluated and the ratio between NCDase and β-actin was calculated and depicted as relative NCDase expression in a graph. Data in **a** and **b** are expressed as percentage of control values and are means ± s.e.m. ($n = 3$). *$P < 0.05$, **$P < 0.01$ considered statistically significant when compared with the vehicle-stimulated values. The inset in **b** shows one representative immunoblot.

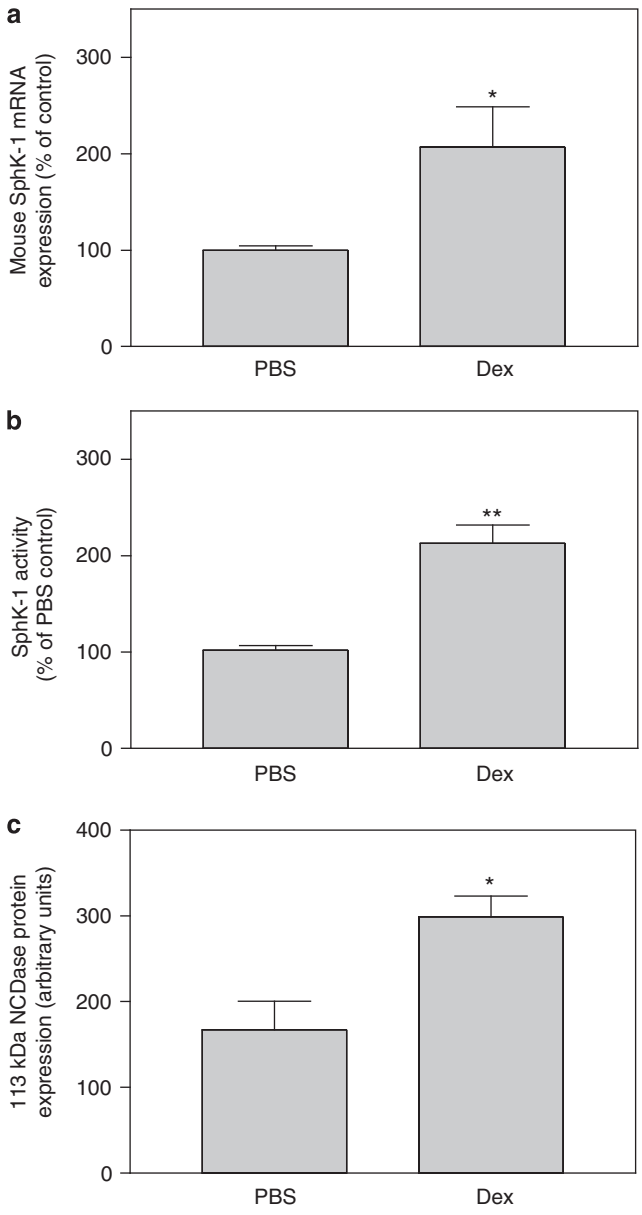

Figure 6 | *In vivo* effect of dexamethasone on sphingosine kinase (SphK1) mRNA and activity, and neutral ceramidase (NCDase) expression in mice. C57BL6 mice were injected intraperitoneally (i.p.) with either phosphate-buffered saline (PBS) as control, or with dexamethasone (dex, 10 mg/kg) for 24 h. Thereafter, kidneys were harvested and taken for isolation of glomeruli by using a differential sieving method. (**a**) RNA was extracted and taken for quantitative PCR analysis of SphK1 and 18S RNA. Protein lysates were taken for (**b**) SphK1 activity or for (**c**) NCDase protein expression by western blot analysis. Results are expressed as percentage of control and are means ± s.d. ($n = 5$). *$P < 0.05$, **$P < 0.01$ considered statistically significant compared with phosphate-buffered saline (PBS)-treated mouse values by a two-tailed *t*-test.

members of the Bcl-2 family such as Bcl-x_s and Bak[36,37] and (2) the downregulation of anti-apoptotic factors such as Bcl-x_L and members of the inhibitor of apoptosis protein family evoked by TNF-α,[37,38] the detailed anti-apoptotic mechanism of glucocorticoids, particularly the signaling pathways involved, still remains to be fully elucidated.

In this study, we show for the first time that glucocorticoids have a protective effect on stress-induced apoptosis of glomerular mesangial cells. This action crucially involves an activation of the sphingomyelin signaling module accompanied by a coordinated expression and stimulation of NCDase and SphK1 activities, which in turn result in increased cellular S1P levels and protection from apoptosis.

Indeed, glucocorticoids have been evaluated as renoprotective compounds in various experimental and clinical

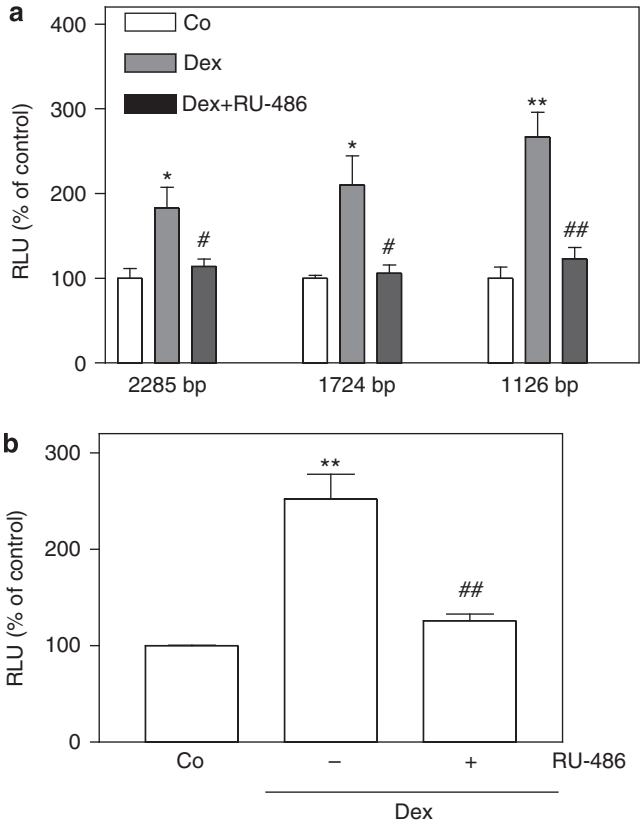

Figure 7 | Effect of dexamethasone and RU-486 on rat neutral ceramidase (NCDase) promoter activity in renal mesangial cells and fibroblasts. (**a**) Normal rat kidney fibroblasts were co-transfected for 6 h with either the 2285, 1724, or 1126 bp fragment of the rat NCDase promoter plus the plasmid coding for *Renilla* luciferase. (**b**) Rat mesangial cells were co-transfected for 6 h with the 2285 bp fragment plus the plasmid coding for *Renilla* luciferase. Cells were then stimulated for 20 h with either vehicle (Co), dexamethasone (Dex, 100 nM), or dexamethasone plus RU-486 (Dex + RU-486; 1 μM) as indicated. The ratio between firefly and *Renilla* luciferase activity was calculated. Relative luciferase activities are expressed as fold induction of control values and are means ± s.d. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ considered statistically significant when compared with the vehicle-stimulated values. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ considered statistically significant when compared with the dexa-stimulated values.

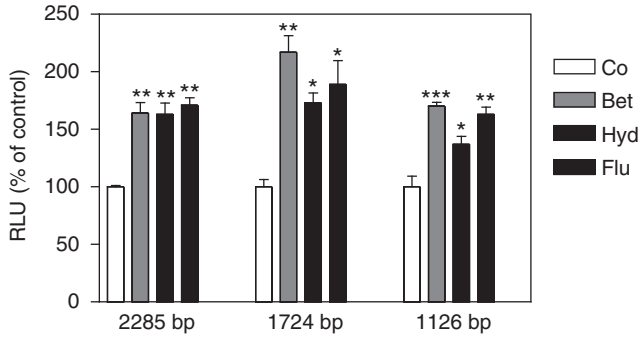

Figure 8 | Effect of various glucocorticoids on rat neutral ceramidase (NCDase) promoter activity. Normal rat kidney fibroblasts were co-transfected for 6 h with either the 2285, 1724, or 1126 bp fragment of the rat NCDase promoter plus the plasmid coding for *Renilla* luciferase, and then stimulated for 20 h with either vehicle (Co) or 100 nM of betamethasone (bet), hydrocortisone (hyd), or fludrocortisone (flu). The ratio between firefly and *Renilla* luciferase activity was calculated. Relative luciferase activities are expressed as percentage of control values and are means ± s.d. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ considered statistically significant when compared with the vehicle-stimulated values.

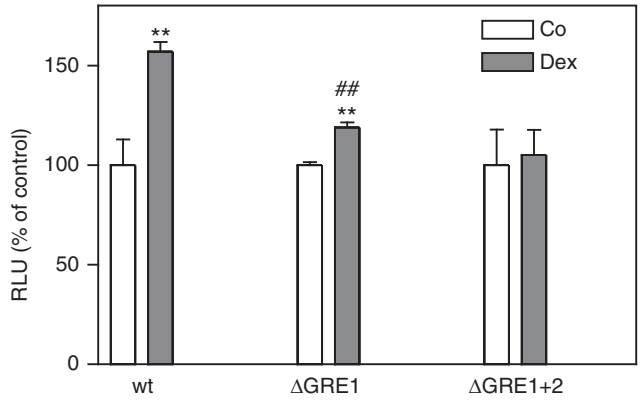

Figure 9 | Effect of glucocorticoid-responsive element (GRE) deletions on dexamethasone-stimulated neutral ceramidase (NCDase) promoter activity. Normal rat kidney fibroblasts were co-transfected for 6 h with the wild-type 1724 bp construct (wt), the GRE1 mutated construct (ΔGRE1), or the GRE1 plus GRE2 mutated construct (ΔGRE1 + 2) as described in the Materials and Methods section. Thereafter, cells were stimulated for 16 h with either vehicle (Co) or dexamethasone (Dex, 100 nM). The ratio between firefly and *Renilla* luciferase activity was calculated. Relative luciferase activities are expressed as percentage of the respective control values and are means ± s.d. ($n = 3$–4). $^{**}P < 0.01$ considered statistically significant when compared with the vehicle-treated wt values; $^{\#\#}P < 0.01$ compared with the dex-treated wt values.

settings.[39,40] They also exert cardio- and neuroprotective actions in the course of ischemic injury by triggering the augmentation of blood flow.[41,42] The latter effects were suggested to be due to a phosphorylation of endothelial nitric oxide synthase by the phosphatidylinositol 3 kinase/protein kinase B pathway.

Dexamethasone was reported to have a direct protective effect on glomerular endothelial cells exposed to TNF-α or lipopolysaccharide,[33] and prevents apoptosis of podocytes stimulated with puromycin.[34] That this protection also extends to mesangial cells, as reported in this study, may explain why glucocorticoids have been used successfully to treat numerous clinical conditions affecting the kidney glomerulus.

Collectively, these observations provide strong evidence that targeting of resident glomerular cells may be as important for the therapeutic action of glucocorticoids as the anti-inflammatory and immunosuppressive action on lymphocytes.

Typically, glucocorticoids bind to the ligand-binding domain of the GR to initiate its nuclear translocation and subsequent interaction with conserved palindromic DNA sequences denominated as GREs. Binding to these cis-acting

promoter elements leads to transcriptional activation of glucocorticoid-responsive genes ('transactivation'). In contrast to transactivation, most inhibitory actions exerted by glucocorticoids arise from the mutual interaction between the GR and transcriptional activators such as activator protein 1 or nuclear factor-κB. This interaction occurs independent from DNA binding and is termed 'transrepression'.[43,44] Pharmacologists use this information to separate transrepression from transactivation to yield the so-called dissociated glucocorticoids that may allow to reduce the side effects of an anti-inflammatory therapy with glucocorticoids.[45,46]

Despite our extensive knowledge about glucocorticoid-induced gene regulation, the global networks regulated by GR remain unknown. Recently, Phuc Le *et al.*[47] reported on 1300 differentially expressed genes in mouse liver exposed to glucocorticoids. Subsequently executed genome-wide location analysis on chromatin from the same livers identified more than 300 promoters that are bound by the GR. Finally, 53 genes were identified to be functionally regulated by ligand-occupied GR. The list contains many genes that are known or suspected GR targets, but not many signaling devices were identified, as these usually are expressed at low levels and require only subtle changes in expression levels for functionality. Not surprisingly therefore, NCDase and SphK1 were missed in this study. An alternative powerful approach is differential proteomic analysis that permits identification of protein modifications and of translational regulation that is not addressable by genomic analysis. The latter approach has been used by Ransom *et al.*[48] to identify 92 proteins in murine podocytes that are differentially regulated by dexamethasone. Among these, three proteins known to protect cells from injury were found to be upregulated by dexamethasone, comprising ciliary neurotrophic factor, αB-crystallin, and heat shock protein 27. Again, besides known factors, such as metabolic enzymes, antioxidant enzymes, or actin cytoskeletal proteins, only few signaling devices and no sphingolipid-metabolizing enzymes were detected. It appears clear that such lists always remain incomplete. As glucocorticoids exert different effects on the many organ systems of the body, it is highly likely that the functional targets of GR are different in each case. In addition, glucocorticoids exert many posttranscriptional and posttranslational actions[49] and show further nongenomic modes of action[50] that alter protein functions profoundly.

In contrast, our strategy of identifying glucocorticoid targets was hypothesis driven. Considering the central role of the sphingomyelin signaling module in apoptosis and cell survival in many different cellular systems, we felt that it is worthwhile to analyze whether glucocorticoids alter the expression of any of the key regulators of this pathway. Especially the two sphingolipids, ceramide and S1P, have been attributed a key regulatory function in the cell by building a so-called cellular rheostat that determines cell growth and death.[3] In addition, in terms of inflammatory mechanisms the balance between ceramide and S1P appears to be a critical factor that determines a pro- or anti-inflammatory outcome.[51] Strikingly, we observed in renal mesangial cells that glucocorticoids coordinately upregulated the S1P-generating enzymes, NCDase and SphK1. The subsequent marked increase in the protective mediator S1P delivered survival signals to the cells and reduced TNF-α/cycloheximide- or staurosporine-triggered apoptosis. Blocking SphK1 activity by the selective inhibitor, SKI II, reverted the protective effects of dexamethasone, suggesting that generation of S1P is a crucial step in the anti-apoptotic action of glucocorticoids. In addition, when mouse mesangial cells were used that had genetically knocked out the SphK1, not only the staurosporine effect on apoptosis was accelerated, but also the protective effect of dexamethasone was blunted.

The transcriptional regulation of NCDase expression by glucocorticoids reported in this study can be attributed to two GREs in the promoter region at –1337 bp and at –890 bp within the NCDase gene. This is obvious from deletional and mutational analysis of various promoter constructs (Figures 7–9) and further supported by the action of the GR antagonist RU-486 (Figure 7a and b). Whether SphK1 expression is also driven by a classical GR/GRE interaction remains to be established in future investigations.

Collectively, our data reveal precise and valuable details of how glucocorticoids affect a major signaling device, the sphingolipid rheostat, that is used in renal cells to switch between survival and death signals. Expressional regulation of signaling molecules, such as NCDase and SphK1, by rather conventional modes through GR/GRE-driven gene expression may have long-lasting effects on cellular behavior that complement rapid non-genomic signaling events evoked by glucocorticoids.[50] This study offers new facets of action of long-known drugs that may provide ways to better understand what the term 'pleiotropic' means in the context of glucocorticoids' action. Eventually, this may also help to pave the way for designing novel SEGRAs (selective glucocorticoid receptor agonists) with an improved benefit-to-risk ratio.

MATERIALS AND METHODS

Chemicals

All glucocorticoids, mineralocorticoids, RU-486, SKI II, staurosporine, and cycloheximide, and TRI-reagent were from Sigma-Aldrich Fine Chemicals (Deisenhofen, Germany); NBD-coupled C12-ceramide (N-C12-NBD-D-erythro-sphingosine) was from Biozol Diagnostica Vertrieb GmbH (Eching, Germany); and Lipofectamine and all cell culture nutrients were from Gibco Invitrogen (Karlsruhe, Germany).

Generation of antibodies

The antibodies against neutral ceramidase and SphK1 were generated and characterized as previously described.[17,23]

Cell culture

Rat renal mesangial cells were isolated and characterized as described previously.[52,53] For the experiments in this study, passages 9–20 were used. NRK fibroblasts (American Type Culture Collection, Rockville, MD) were cultured in modified Eagle's minimum

essential medium supplemented with 5% fetal calf serum and antibiotics. Mouse mesangial cells were isolated from kidneys of C57BL6 mice or SphK1-deficient mice and cultivated as previously described.[28]

Cell stimulation and western blot analysis

Cells were stimulated with the indicated substances in Dulbecco's modified Eagle medium containing 0.1 mg/ml of fatty acid-free bovine serum albumin. To stop the reaction, the medium was removed, and the cells were homogenized and processed exactly as previously described.[17] Lysates, 50–100 µg, were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, and western blot analysis was performed as previously described.[17,23]

Sphingolipid quantification by liquid chromatography-coupled mass spectrometry

In six-well plates, 10^6 cells were stimulated as indicated. Cell monolayers were then scraped in pure methanol containing an internal C17-S1P standard and subjected to lipid extraction as described by Bligh and Dyer.[54] Lipids were redissolved in dimethylsulfoxide containing 2% HCl and taken for liquid chromatography-coupled mass spectrometry analysis exactly as previously described.[28]

NCDase activity assay

Stimulated mesangial cells were scraped and lysed as described above. The NCDase activity assay was performed according to Galadari *et al.*[55] A total of 100 µg protein lysate dissolved in 200 µl lysis buffer was incubated with 0.2 µmol NBD-coupled C12-ceramide (N-C12-NBD-D-erythro-sphingosine) for 2 h at 37 °C. Reaction was stopped by addition of 200 µl chloroform/methanol (1:1, v/v). For phase separation, samples were centrifuged for 10 min at 13 000 *g* and 100 µl of the organic phase was taken and dried using a vacuum concentrator. Lipids were dissolved in 25 µl chloroform/methanol (2:1, v/v) and spotted on to thin-layer chromatography plates. The cleaved product NBD-dodecanoic acid was separated by developing the plate in chloroform/methanol/25% ammonium hydroxide (90:30:0.5, v/v). The thin-layer chromatography plate was scanned using PharosFX (Bio-Rad, Munich, Germany) and C-12 NBD-fatty acids were analyzed using Quantity One 1-D Analysis Software.

Sphingosine kinase activity assay

In vitro SphK1 activity assay was performed exactly as described previously.[23]

Cell transfections and NCDase promoter studies

Different NCDase promoter fragments with sizes of 2285, 1724, and 1126 bp were cloned from rat genomic DNA (Novagen, Darmstadt, Germany) by PCR using the same reverse primer: 5′-CCGCTCGAG GAGTCCAAATG-3′ and the following forward primers: 2285 bp: 5′-CCGACGGGTGATGAAGCTG-3′; 1724 bp: 5′-CCGACGCGTCTAG CACGTGTG-3′; and 1126 bp: 5′-CCGACGCGTGTTTGCTACTAT GAC-3′. The NCDase promoter fragments were fused into the luciferase reporter gene-containing vector pGL3 (Promega, Mannheim, Germany). NRK or rat mesangial cells were cultured in 12-well plates and transfected in serum-free media with 150 ng of reporter plasmid DNA and 30 ng of Renilla luciferase reporter plasmid using Lipofectamine (fibroblasts) or Effectene (mesangial cells). After 6 h, the cells were stimulated as indicated in the figure legends. To measure NCDase promoter activity, a luciferase reporter gene assay was performed using a Lumat LB9507 luminometer (Berthold Detection Systems, Pforzheim, Germany). Values for NCDase promoter activity were calculated from the ratio of firefly to *Renilla* luciferase activities and expressed as a percentage of control.

Site-directed mutagenesis of the NCDase promoter

Site-directed mutagenesis of two putative GREs, GRE1 (mutation of –1339 to –1336 bp) and GRE2 (mutation of −891 to –889 bp), in the NCDase promoter was carried out with the 'QuickChange Site-Directed Mutagenesis Kit' (Stratagene Agilent Technologies, Waldbronn, Germany). The primer pair (mutation sites are underlined) used for ΔGRE1 was forward: 5′-CAGGTCTCAAAGA CCAAAGAAAGACATTAGCATCCTGATGTAACAGGAAAG-3′ and reverse: 5′-CTTTCCTGTTACATCAGGATGCTAATGTCTTTCTTTG GTCTTTGAGACCTG-3′; and the primer pair for ΔGRE2 was forward: 5′-TCCTCTTCACAGCTGCTTAGACATCAGTCTCCAAG AGGAGGAAAA-3′ and reverse: 5′-TTTTCCTCCTCTTGGAGAC TGATGTCTAAGCAGCTGTGAAGAGGA-3′. The mutations were confirmed by DNA sequencing.

Quantitative real-time PCR (TaqMan)

Total RNA, 1 µg, isolated with TRI-Reagent was used for reverse transcriptase-PCR (RevertAid First strand synthesis kit, Fermentas, St Leon–Roth, Germany) using an oligo (dT) primer for amplification. The real-time PCR reaction was carried out in 96-well PCR plates from Applied Biosystems Applera (Darmstadt, Germany). Probes and primers for rat NCDase, SphK1, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were obtained from Applied Biosystems. The reporter dyes chosen were 6-FAM and VIC. As PCR-Mix, Absolute QPCR low Rox Mix from Abgene (Dreieich, Germany) was used. The run was performed on the Applied Biosystems 7500 Fast Real-Time PCR System. The cycling conditions were as follows: 95 °C for 15 min (1 cycle), 95 °C for 15 s, and 60 °C for 1 min (40 cycles). 7500 Fast System SDS Software from Applied Biosystems was used to analyze real-time and end point fluorescence.

Determination of DNA fragmentation

Apoptosis was detected by measuring the appearance of cytoplasmic histone-associated DNA fragments by photometric enzyme-linked immunosorbent assay (Roche Diagnostics, Mannheim, Germany). The preparation of cell lysates and the assay procedures were performed according to the manufacturer's protocol. Alternatively, the amount of apoptotic cells was quantified by flow cytometric analysis of DNA fragments residing in the subG1 phase. In brief, stimulated cells were detached by trypsin/EDTA and fixed in 70% ethanol for 16 h at −20 °C. Cells were washed with 38 mM sodium citrate and incubated for 30 min with 10 µg/ml propidium iodide and 10 µg/ml RNase A. Cells were analyzed by flow cytometry for DNA content using a FACS Calibur (Becton & Dickinson, San Jose, CA, USA).

Animal experiments

All animal experiments were conducted in accordance with the German Animal Protection Law and were approved by the ethics review committee of the District Governments of Darmstadt, Germany. Male C57BL6 mice, 2 months old, were intraperitoneally injected with dexamethasone (10 mg/kg) dissolved in phosphate-buffered saline. Control animals were injected with 150 µl of phosphate-buffered saline. After 24 h, kidneys ($n = 5$ per group)

were harvested and taken for isolation of glomeruli by using a differential sieving method.

Statistical analysis

Statistical analysis was performed using either a two-tailed *t*-test or one-way analysis of variance followed by Bonferroni's *post hoc* test for multiple comparisons (GraphPad, InStat version 3.00 for Windows NT, GraphPad Software, San Diego, CA, USA).

DISCLOSURE

All the authors declared no competing interests.

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